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# METHOD FOR STIMULATING FIBRINOLYTIC EFFECT

## Field of Invention

This invention relates to a method for stimulating the fibrinolytic effect in vivo. More particularly this invention relates to a method for stimulating the release of natural reserves of tissue plasminogen activator (t-PA) in vivo.

## Background of Invention

In normal hemostasis, which may be defined as the arrest of blood flow from a leaking blood vessel by a normal physiological process, the primary event is platelet plug formation followed by fibrin generation. Subsequent to the deposition of the fibrin the fibrinolytic system orchestrates its orderly removal as the permanent repair process ensues. The fibrinolytic system is a physiological process whereby activators of a zymogen plasminogen convert this to an active enzyme plasmin. This enzyme degrades fibrin which is the final product of the coagulation process. In contrast, thrombosis is an abnormal, uncontrolled, unregulated clotting mechanism which takes place inside an intact blood vessel and which compromises the welfare, and frequently the life, of the organism by innappropriate blockage of the blood flow. In thrombosis the primary event is exaggerated fibrin generation which compromises the flow of blood to such vital organs as the heart and brain. In pathological situations the

fibrinolytic system may be impaired or unable to cope with the degree of fibrin generation.

Recently, significant advances have been made in isolating, characterizing and synthesizing by biotechnological techniques a specific plasminogen activator (t-PA) and it has been demonstrated that infusion of high doses may achieve accelerated lysis of thrombi forming during such conditions as myocardial infarction. Similar effects have also been demonstrated with streptokinase, an enzyme extracted from the Daeteria streptococci and urokinase, a plasminogen activator present in urine. These agents and particularly t-PA thereapy are, however, extremely costly.

Object of Invention

It is an object of the present invention to provide an alternative method for stimulating the fibrinolytic effect which would find application in the treatment and management of patients who have suffered acute myocardial infarction and other conditions where the blockage of a blood vessel by a thrombus plays a pivotal role in the genesis of the condition.

It is another object of the present invention to provide an alternative to the existing plasminogen activator therapy.

Statement of Invention

By one aspect of this invention there is provided a method for endogenously stimulating a fibrinolytic effect in vivo comprising intravenously administering to a patient an

effective amount of a mixture of blood Factor Xa and phospholipid vesicles.

Brief Description of Drawings

Fig. 1 is a graph illustrating fibrinogen and t-PA levels in normal and haemophilic dogs after treatment with Factor Xa/PCPS at $6.5 \times 10^{-12}$ and $4.0 \times 10^{-7}$ moles/kg body weight.

Fig. 2 is a graph illustrating fibrinogen and t-PA levels in normal and haemophilic dogs after treatment with Factor Xa/PCPS at $2.6 \times 10^{-11}$ and $4.0 \times 10^{-8}$ mole/kg body weight.

Figs. 3a, b & c are graphs illustrating plasma t-PA levels in chimpanzees after treatment with Factor Xa/PCPS at various dosage levels (IU/ml).

Figs. 4a, b & c are graphs illustrating fibrinogen levels in chimpanzees after treatment with Factor Xa/PCPS at various dosage levels (g/l).

Fig. 5 is a graph illustrating dose response relationships between Xa/PCPS and fibrinogen comsumption and peak levels of t-PA.

Fig. 6 is a schematic diagram illustrating the stages in the cascade process for blood coagulation.

Detailed Description of Preferred Embodiments

It has been previously demonstrated that a combination of phosphatidyl choline-phosphatidyl serine lipid vesicles when infused with factor Xa bypasses factor VIII in the

classic cascade for blood coagulation in vivo illustrated in Fig. 6.

Attention is directed to U.S. patent 4,610,880 and 4,721,618 issued 9 September 1986 and 26 January 1988 respectively and assigned to the assignee of the present application the disclosures of which are incorporated herein by reference. Factor Xa/PCPS has been demonstrated to be an effective composition for controlling bleeding in both hemophilia patients, who are generally deficient in Factor VIII, and in normal patients, when given in critical dosage forms. It has also been shown that the combination of factor Xa and PCPS is thrombogenic in a stasis model in rabbits (Blood 59, 401-407, 1982) and produces clinical and laboratory evidence of disseminiated intravascular coagulation in dogs similar to that seen following the use of prothrombin complex concentrates in man (J. Clin. Invest. 74, 2219-2225, 1984). Consequently, in order to achieve hemostasis a critical dose ratio of Factor Xa to PCPS in required. Above a given level, unnacceptable toxicity (thrombogenicity) occurs whereas below a certain level a hemorrhagic tendency is produced in normal ie. VIII:C replete, animals presumably due to the relative excess of phospholipid favouring the anticoagulant effect of activated Protein C.

Factor Xa may be obtained by fractionating normal mammalian plasma to obtain the precursor zymogen Factor X which can be activated by known procedures (Bajaj et al. J.

Biol. Chem. 248: 7729, 1973, Downing et al. J. Biol. Chem. 250: 8897, 1975.

Example 1

**Coagulation studies in normal and haemophilic dogs.**

**Animals.** The studies were performed on either normal mongrel dogs, provided by the Queen's University animal care facility, or purebred/crossbred haemophlic (factor VIII deficient) animals raised in the Queen's University haemophlic dog colony (Giles et al. Blood 60: 727-730, 1982). Where necessary these animals were treated with canine cryoprecipitate prepared from fresh canine whole blood by standard blood banking techniques.

The cutical bleeding time (CBT) was performed as previously described in the Giles et al. reference above. In brief, the animals were lightly anaesthetized and the apex of a nail cuticle severed using a guillotine device. The cuticle bleeding time is the time from injury induction to the cessation of bleeding. In normal animals, the cuticle bleeding time is 6.0 $\pm$ 3.7 (SD) min (n=15). In factor VIII deficient animals, bleeding usually continues until arrested by local cautery by silver nitrate applicators or factor VIII replacement with cryoprecipitate. Correction of CBT usually occurs in haemophilic animals when the factor VIII level is restored to > 25%.

**Coagulation and fibrinolytic assays.** Blood was collected by venipuncture using a 21-gauge butterfly needle (Abbott-Ireland, Sligo, Ireland) and a two syringe

technique. The anticoagulant used was sodium citrate (3.8% wt/vol); 9 vol of blood to 1 vol anticoagulant in plastic tubes. Platelet-poor plasma was separated by centrifugation at 2500 g for 15 min at 4°C. In most cases the plasma was snap frozen and stored at -70°C until processed as decribed below.

Factors V and VIII were assayed by one-stage assay. In each case, a normal pool plasma obtained from 20 normal dogs was used as a reference standard (Giles et al. 1984). Fibrinogen was measured as the thrombin-clottable protein by the method of Clauss (Clauss, 1957) using a fibrometer (BBL Microbiology Systems Division, Beckton Dickinson Co., Cockeysville, Md.) and purified bovine thrombin. Platelet counts were measured in EDTA anticoagulated whole blood using a Super-S particle counter (Coulter Electronic Inc., Hialeah, Fla.). Plasminogen activator was measured as lysine adsorbable plasminogen activator (LAPA) by a modification of the method of Comp et al. (1981) as follows: 0.5 ml of platelet poor plasma was applied to 1 ml of lysine sepharose packed in 10 ml disposable plastic columns. The column was then washed with 6 ml Tris/EDTA buffer (50 mM Tris, 1 mM EDTA, 1 mM benzamidine HCl, 0.05% Tween 20) pH 7.4. LAPA was eluted with 0.5 M $NH_4SCN$ made up in the wash buffer. The collection volume was 2.0 ml. The eluted LAPA was assayed on fibrin plates following the addition of the sample (12.5 l) together with plasminogen (12.5 l to 2 mg/ml). The plates were read after 20 h incubation at 37°C.

Each plate included dilutions of human melanoma tissue type plasminogen activator as a reference standard from which a standard curve was constructed.

Bovine factor Xa and prothrombin were prepared by the method of Bajaj & Mann (1973). Factor X was activated by immobilized purified Rusell viper venom by the method of Downing et al. (1975). The concentrated stock solution of factor Xa (1 mg/ml) was assayed as previously described (Giles et al., 1982) and stored in 50% glycerol at -20°C and diluted with tris-HCl (0.02 M) buffered saline (0.15 M), pH 7.4, containing polyethylene glycol (1%) to the prescribed dose (see below) just before use. PCPS lipid vesicles were prepared and assayed as previously described (Nesheim et al., 1979). The PCPS lipid vesicles were stored at 4°C and used within 10 days of preparation. Protein C was prepared and activated with purified thrombin as described by Kisiel & Davies (1981). Plasminogen was prepared according to the method of Powell & Castellino (1981). Fibrinogen was prepared according to the method of Straugn & Wagner (1967) and depleted of plasminogen by affinity chromatography on lysine sepharose.

**Coagulation studies.** Blood samples were obtained as previously described prior to and at various time intervals following the infusion of factor Xa/PCPS. Results in typical experiments of assays on factors V and VIII, fibrinogen and plasminogen activator are shown in Figs. 1 and 2. The data shown in Fig. 1 was obtained using the dose

of factor Xa/PCPS that did not correct the CBT in the haemophilic animals. Equivalent data obtained using the factor VIII bypassing dose are shown in Fig. 2. With both dosage regimens significant falls in factors V and VIII and fibrinogen occurred. Of particular interest, the factor VIII bypassing dosage was associated with a notable increase in the levels of circulating lysing adsorbable plasminogen activator. In contrast the changes noted with the alternative dosage regime were unremarkable.

Example 2

**Coagulation studies in primates**

**Species.** All the studies described were performed in adult chimpanzees (pan troglydytes) of both sexes. Their body weight ranged from 45 to 68 kilograms. The animals are members of the permanent chimpanzee colony maintained at the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP), New York Medical Centre, Tuxedo, New York. All the animal had previously been screened for any evidence of a hemostatic abnormality and not been used for any experimental purpose for at least six months prior to the performance of the study. They were maintained on a standard primate diet but were fasted for 12 hours prior to study. The study protocol used had received prior approval by the LEMSIP Institutional Animal Care and Use Committee.

**Experimental Procedure.** Each animal was anaesthetized with ketamine (10 mg/kg body weight). Venous blood samples

were obtained during the experimental period via a venous catheter placed in a superficial cubital vein. The catheter was kept open between blood sampling by a continuous, slow infusion of isotonic saline for injection. Following anaesthetic induction, an equilibration period of 60 minutes was allowed before the test material (see later) was infused as a slow bolus (>30 sec.). Each dosage regimen (see later) was given to one animal. Blood samples were taken immediately after anaesthetic induction (-60 min.) and 5 min. before infusion of the test material (-5 min.). Post-infusion samples were obtained at 2, 5, 10, 15, 20, 30, 45, 60 and 90 minutes. Each animal was monitored clinically together with continuous recordings of EKG and blood pressure. Particular note of evidence of bleeding at the site of venipunctures, etc., was taken.

**Dose of Test Material.** Factor Xa and PCPS vesicles were administered at four dosage regimens, ranging from 12.25 pMoles/kg to 36.60 pMoles/kg and from 18.85 nMoles/kg to 56.30 nMoles/kg, respectively. In each case, the ratio of the dose of factor Xa/PCPS was maintained at 0.65 (pMole F.Xa/nMole PCPS). In order to determine an indicator of thrombin generating potential of each dose combination, each was added to normal pooled donor plasma, that had been dialized against 0.02 HEPES, 0.15 M NaCl to remove the anticoagulant, and then the clotting time determined following recalcification. The volume of procoagulant material added was adjusted to allow for the estimated

dilution that would occur in vivo assuming a constant relationship between plasma volume and body weight (35 ml/kg). The four dosages used produced in vitro plasma clotting times of 15, 20, 25, and 30 seconds. It should be noted that the precise dosage administered to each animal was given on a dose per body weight adjusted basis. Each component, in the form of a stabilized, lyophilized preparation, was reconstituted with water for injection, then mixed at the appropriate dosage immediately before infusion.

**Coagulation and Fibrinolytic Testing.** All samples were obtained using a two syringe technique. Blood was immediately anticoagulated using either buffered citrate (0.04 M citric acid/0.06 M sod. citrate) alone for coagulation and functional t-PA assays (9 vols blood/1 vol anticoagulant) or sodium citrate (10mM) plus PPACK (20 M) for the remaining fibrinolytic assays. Platelet poor plasma was prepared immediately be centrifugation at 2500 G for 30 mins. Plasma samples were snap frozen, stored at -80°C and rethawed immediately before assay. Assays of factors V and VIII were performed as previously described using human reagents. Thrombin clotting times were performed as previously described. Fibrinogen was assayed by the method of Clauss and fibrin degradation products by the method of Merskey. Alpha-2 antiplasmin was assayed chromagenically using the chromagenic substrate S-2251, according to the method of Edy. Tissue plasminogen activator was measured

functionally by the method of Verheijen et al and antigenically by the method of Holvoet employing the International t-PA standard. Functional tissue plasminogen activator inhibitor was measured by the method of Verheijen. u-PA was measured by the method of Darras et al. D-dimer was measured by ELISA, using the Dimer Test (American Diagnostic Inc., Greenwich, CT). B-beta 1-42 was measured using reagents obtained from the New York Blood Centre.

**Materials and Reagents.** All reagents used were reagent grade or better. Factor X was prepared from heat treated human prothrombin complex concentrate (Profilnine$^{R}$ Alpha Therapeutic Corp., Los Angeles, CA) and activated with immobilized Russell viper's venom in the presence of calcium. Non-activated zymogen and any contaminating RVV activator were removed by chromatography on benzamidine Sepharose. Homogeneous factor Xa was then stabilized with human serum albumin (3.5 mg/mg F.Xa) and polyethylene glycol - 4000 (35 mg/mg F.Xa) prior to lyophilization. The specific activity of the preparation used was 1350 u/mg. PCPS vesicles were prepared as previously described by the method of Barenholz et al. as modified by Nesheim et al. type III phosphatidylcholine (egg yolk) and phosphatidyl-serine (bovine brain) were obtained from Sigma Chemical Co., St. Louis, MO. The molar ratio of PC:PC was 3:1. The unilamellar vesicles (size range 60 - 90 nm) were aseptically filled into glass vials and lyophilized following the addition of sucrose (10%) as a stabilizer.

Both the factor Xa and PCPS vesicles were reconstituted with water for injection and mixed at the required dosage immediately before each experiment. Following reconstitution and mixing, the material was drawn into a plastic syringe and kept at room temperature until the time of bolus infusion.

Results

All four dosage regimens were well tolerated with no untoward clinical effects observed during the study or following recovery from anaesthesia. There was no evidence of a bleeding diathesis, such as unusual bleeding from venipuncture sites. Significant falls in both factors V and VIII occurred within two minutes following the infusion of F.Xa/PCPS in agreement with the findings noted in Example 1 for the hemophlic dogs. Concurrent with this observation was clear evidence of thrombin generation and Protein C activation.

The effects of the bolus infusion F.Xa/PCPS on t-PA functional levels and antigen are shown in Figures 3a and 3b, respectively. These parameters began to change almost immediately following the infusion of F.Xa/PCPS; reached a maximum at about 10 minutes; and declined thereafter. A clear dosage effect was evident in these data. Ten minutes after the lowest dose, activity and antigen levels were 12 u/ml and 32 ng/ml. These values are modestly incremented compared to the pre-infusion values of 1 u/ml and 8 ng/ml antigen 10 minutes post-infusion. These latter levels

exceed by more than a factor of 100 the basal levels of activity and antigen. In contrast, levels of u-PA antigen did not change from baseline values during the whole time course of the studies at any dosage.

Figure 3c indicates the time course of relative levels of alpha-2 antiplasmin. The progressive consumption of the inhibitor was evident in each case and the magnitude of the effect mirrored the excursions in levels of t-PA. The magnitudes of the rates of change of alpha-2 antiplasmin were approximately coincident with levels of t-PA, suggesting that the appearance of t-PA was accompanied by palsminogen activation. At the highest dose of F.Xa/PCPS, alpha-2 antiplasmin levels fell to about 30-40% of normal within the first 10 minutes following infusion and then stabilized at that level over the remaining 90 minutes of the experiment.

The time course of levels of fibrinogen, FDP, D-dimer and B-beta 1-42 following the bolus infusion of F.Xa/PCPS are shown in Figures 4a-d. A dosage effect was again observed, in that the two lower doses produced relatively modest changes in these four parameters, compared to the higher doses which produced relatively massive changes. At the highest dose, clottable fibrinogen, 5 minutes after infusion, was less than 10% of the pre-infusion level. By 10 minutes, fibrinogen was no longer detectable and remained so for the entire course of the experiment. As these measurements were made as thrombin clottable fibrinogen, these data may be over-estimates of the true decreases due

to interference with fibrin polymerization resulting from the relatively large increase in FDP (Figure 4). Figure 4 indicates that, within 10 minutes, fibrinogen consumption was approximately 2800 g/ml. The consumed fibrinogen reappeared as FDP levels exceeding 2000 g/ml. In addition, D-dimer levels approached 400 g/ml within 10 minutes. The changes in concentrations of fibrinogen and D-dimer were 7.9 M and 2.2 M, respectively, Since each D-dimer derives from two fibrin(ogen) monomers, D-dimer appeared at a ratio approximately 0.6 mole per mole of fibrinogen consumed. This ratio suggests that the majority of the fibrinogen consumed was converted to cross-linked fibrin, which subsequently yielded D-dimer in response to fibrinolytic activity. Levels of B-beta 1-42 were characterized by a transient peak that corresponded in time with the peak of t-PA activity. The temporal correlation with t-PA levels suggest that B-beta 1-42 reflects fibrinolytic activity, and the transient response reflects a relatively high rate of clearance. The rapid clearance implies the peak values can provide only a lower estimate of the quantity of B-beta 1-42 produced in the first 10 minutes. At the highest dose of Factor Xa/PCPS, B-beta 1-42 was observed at a level of 3.0 M. Since each fibrinogen can yield two B-beta 1-42 fragments, at least 20% of the theoretical yield of B-beta 1-42 was realized. The presence of B-beta 1-42 at these levels suggests that some of the fibrin had been deposited as fibrin I, or that some

fibrinogenolysis had occurred, or both.

The dose response relationships between F.Xa/PCPS, fibrinogen consumption and peak levels of t-PA are shown in Figure 5. The figure includes data from studies using dosages of Factor Xa and PCPS that were varied in terms of both their absolute concentrations and their molar ratios. Thus, the results on the horizontal axis are expressed as $\sqrt{[Xa].[PCPS]}$. This representation was utilized because the magnitude of the effect of the combination of these components changes approximately as the product of their individual levels when assessed by thrombogenicity in a rabbit model. The magnitude of their effect is therefore linear in either one, when the other is held at a fixed level. Thus, the plot shown in Figure 5, in effect represents an approximation to the dose response which would be obtained as a function of the concentration of either component in the presence of a fixed concentration of the other. These data suggest a steeply sigmoidal relationship between the dose of either component and each of the two measured responses, ie. fibrinogen and t-PA. In addition, the two responses appear tightly coupled in that the magnitude of the drop in fibrinogen induced by the procoagulant is accompanied by the appearance of t-PA at a corresponding magnitude. The coupling could occur either by the appearance of t-PA in response to fibrin deposition, or consumption of fibrinogen through fibrinogenolysis mediated by t-PA and plasmin. Since fibrinogen consumption anteceeds somewhat the appearance of t-PA (Figures 3a,b and Figure

4a), and products indicative of fibrinolysis rather than fibrinogenolysis, eg. D-dimer, were present at levels approaching within a factor of two stoichiometric equivalents with the fibrinogen consumed, the data of Figure 5 most likely imply that t-PA appears in response to the fall of fibrinogen and subsequent fibrin deposition. These data strongly imply that mechanisms exist in vivo whereby an immediate and appropriate fibrinolytic response can be mounted to clear inappropriately deposited fibrin.

All plasma samples were also assayed for plasminogen activator inhibitor (PAI-1) but in all cases, although the pre-infusion sample demonstrated some activity, this was at the lower limit of sensitivity of the assay (< 6 IU/ml). In all cases no detectable activity could be measured post-infusion of F.Xa/PCPS but returned at the lowest level detectable at 45 minutes post-infusion in the case of the two lowest doses and at 90 minutes in the case of the two highest dosages. Levels higher than the minimum detectable limit were not recorded on any occasion.

Examples 1 and 2 demonstrate that dogs and primates have a profound ability to mount a fibrinolytic response to a procoagulant stimulus, similar to that which has been demonstrated in man using various stimuli to promote increased availability of plaminogen activator. The magnitude of the response using Xa/PCPS is, however, remarkable and is not believed predictable. It is reasonable to assume that the in vivo generation of thrombin

is central to the events observed although a direct effect of the infused F.Xa and PCPS vesicles cannot be excluded. The response cannot be attributed solely to a direct effect of thrombin, for example in releasing t-PA from the endothelial cell stores, or an indirect effect, for example by the generation of fibrin or the activation of Protein C. In the dog significant activation of Protein C occurs under such circumstances and more recently, it has been confirmed that this also occurs in the chimpanzee. It has been suggested that the fibrinolytic promoting effect of APC observed in vitro or in vivo may result from the complexing of t-PA with its primary natureal inhibitor, PAI-1. Evaluating of this mechanism is complicated by the predictably low levels of PAI-1 in the normal primate and thus the relative insensitivity of the assay available. Nonetheless, the loss of all detectable activity following Xa/PCPS infusion suggesting complexing was of interest but would appear to be an unlikely explanation for the enormous increases in total PA activity that was subsequently observed. These observations suggested a significant increase in the total available t-PA plasma pool rather than in the net functional activity alone. The concurrent major increase in antigen levels supports this contention, ie. a major release of stored t-PA has occurred. As shown in Figure 4, the data are suggestive of the changes in t-PA availability being consequent on fibrin generation and presumably its disseminated deposition on the endothelium. The induction of t-PA release following the deposition of

fibrin on endothelial cells grown in culture has been reported by others. However, thrombin itself has also been shown to induce the same response. The limitations conferred by the complexity of the in vivo response do not allow any firm conclusions in this regard. Similarly, the data does not permit the conclusion that the subsequent overall response was primarily fibrinogenolysis or fibrinolysis or both. The changes in FDP and B-beta 1-42 could be accounted for either by fibrinolysis or fibrinogenolysis. The D-dimer assay is specific for crosslinked fibrin, ie. either fibrin I or II, and the results observed therefore indicate that, at the higher dosages, significant fibrinolysis had occurred. However, calculation of the total quantity of fibrinogen lost could not be accounted for by the quantity of D-dimer generated at any given time. Whether the deficit can be accounted for by concurrent fibrinogenolysis, clearance of fibrin degradation products, sequestrated fibrin or a combination of all three and/or other mechanisms, has not been determined thus far. Nonetheless, the results do clearly demonstrate the magnitude of the intact animal's ability to respond to a procoagulant stimulus in terms of both promoting increased availability of tissue plasminogen activator and the very significant functional capability that this has on the endogenous generation of plasmin and the lytic state that develops subsequently.

We Claim

1. A method for endogenously stimulating a fibrinolytic effect in vivo comprising intravenously administering to a patient an effective amount of a mixture of blood Factor Xa and phospholipid vesicles.

2. A method for stimulating release of natural reserves of tissue plasminogen activator in vivo, comprising intravenouly administering to a patient an effective amount of blood Factor Xa and phospholipid vesicles.

3. Method as claimed in claim 1 wherein said phospholipid vesicles comprise phosphatidyl choline and phosphatidyl serine.

4. A method as claimed in claim 2 wherein said phospholipid vesicles comprise phosphatidyl choline and phosphatidyl serine.

5. A method as claimed in claim 3 wherein the ratio of said Factor Xa, in pMoles/kg body weight to said phospholipid in nMoles/kg body weight is 0.65.

6. A method as claimed in claim 4 wherein the ratio of said Factor Xa, in pMoles/kg body weight to said phospholipid in nMoles/kg body weight is 0.65.

7. A method as claimed in claim 5 wherein the dose of said Factor Xa is between 12.25 and 24.5 pMoles/kg body weight and the dose of said phospholipid is between 18.85 and 37.7 nMoles/kg body weight.

8. A method as claimed in claim 6 wherein the dose of said Factor Xa is between 12.25 and 24.5 pMoles/kg body weight and the dose of said phospholipid is between 18.85 and 37.7 nMoles/kg body weight.

9. A method as claimed in claim 7 wherein said phospholipid vesicles are lyopholized.

10. A method as claimed in claim 8 wherein said phospholipid vesicles are lyopholized.

11. A method as claimed in in claim 8 wherein said Factor Xa is lyopholized.

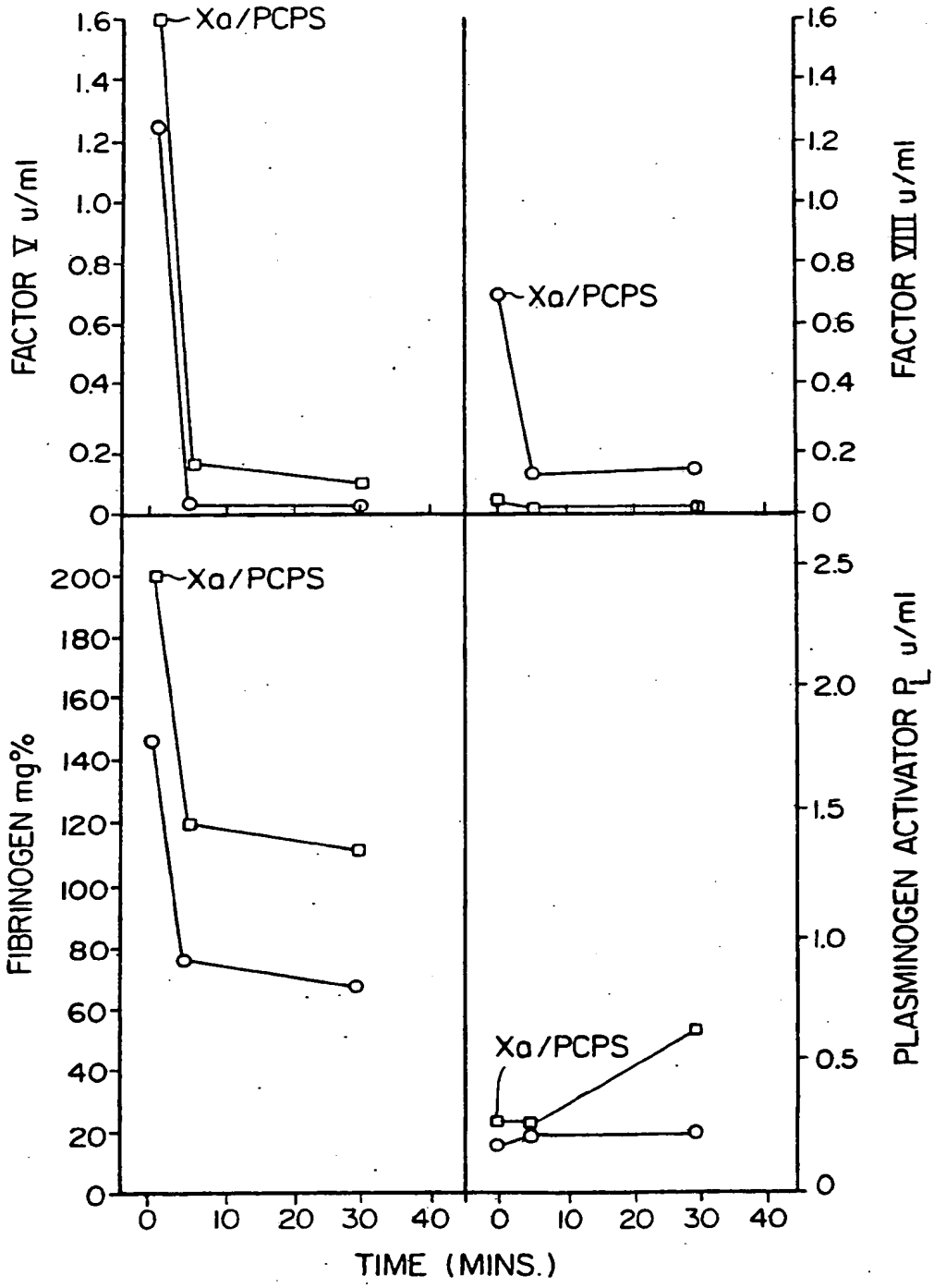


FIG. 1

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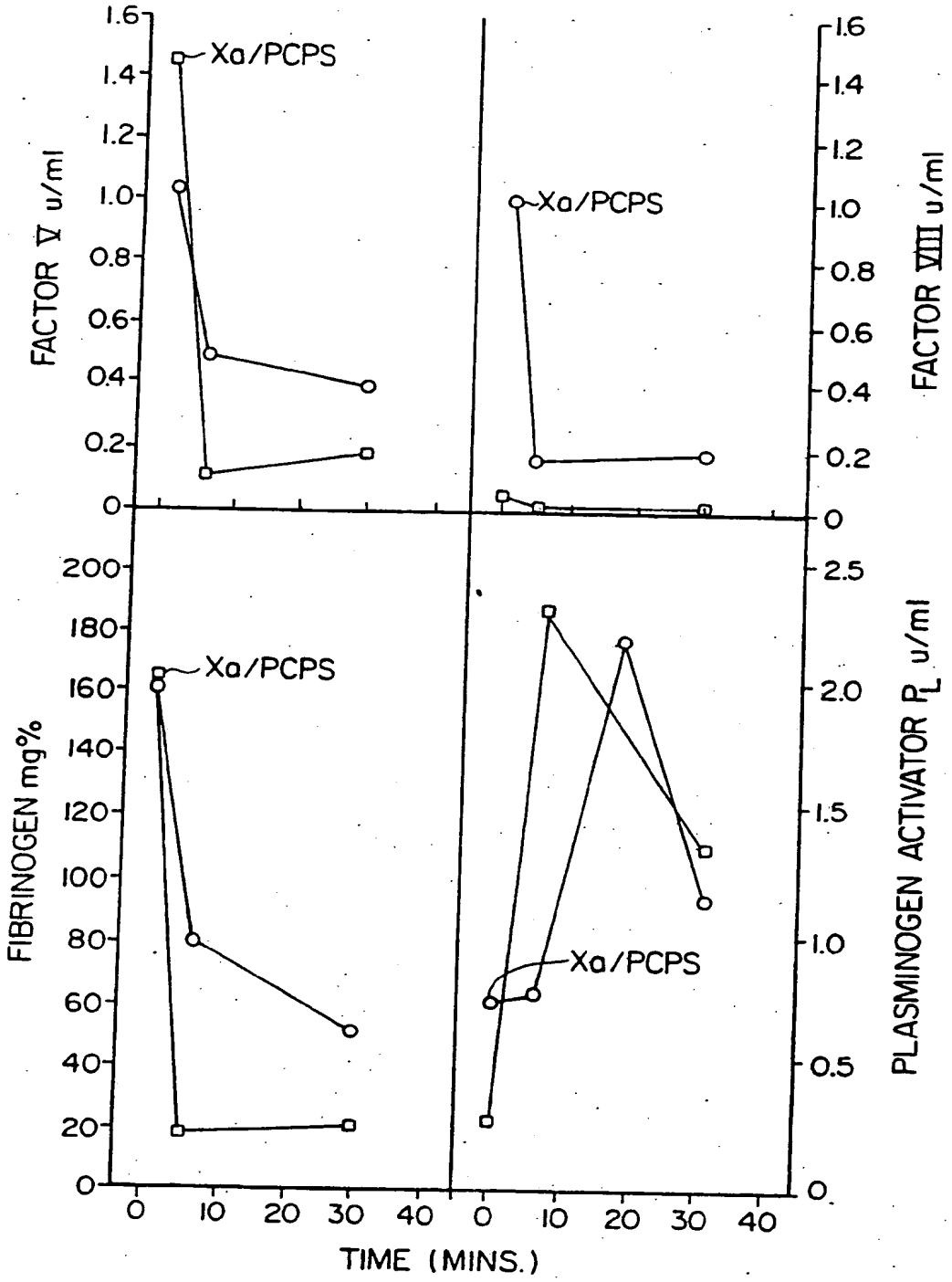



FIG. 2

FIG. 3a

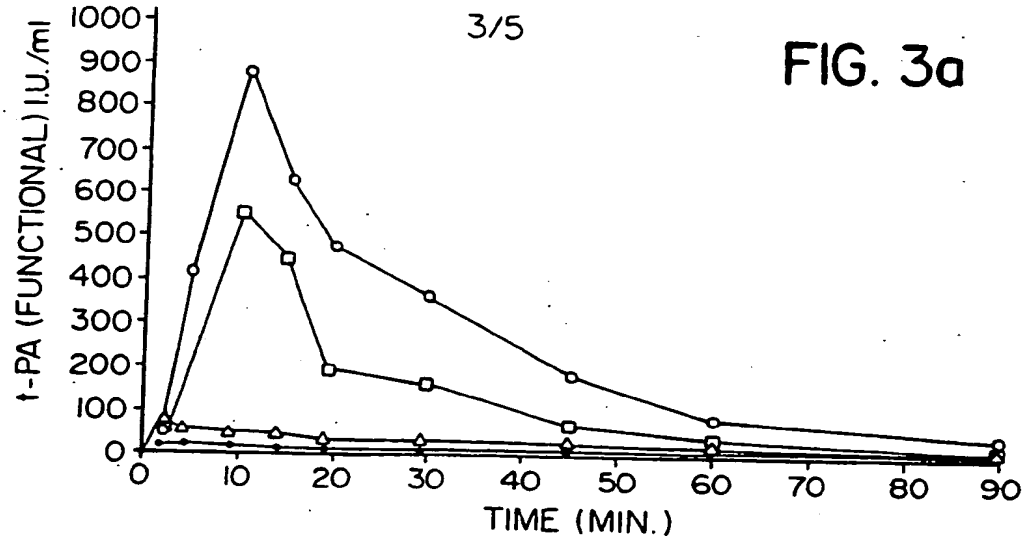



FIG. 3b

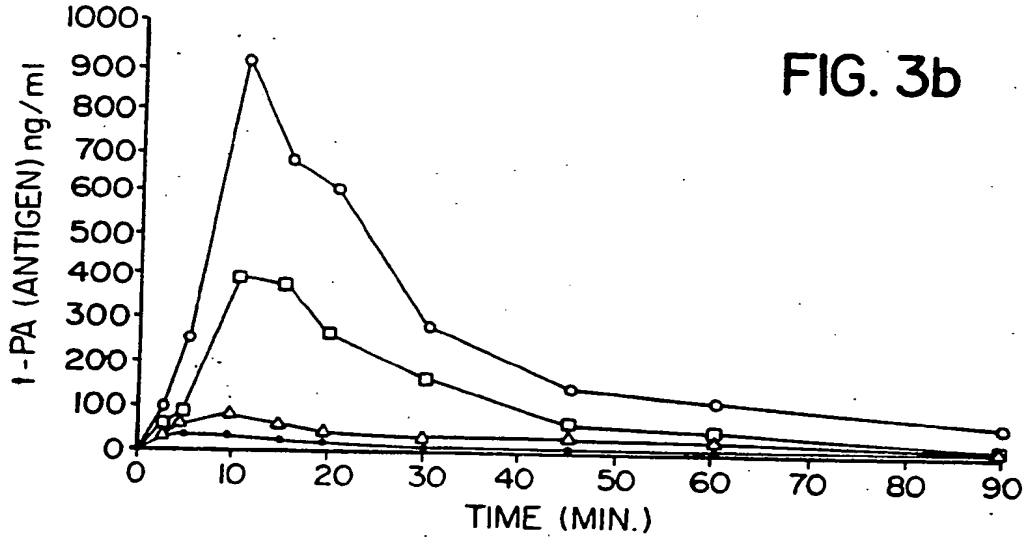
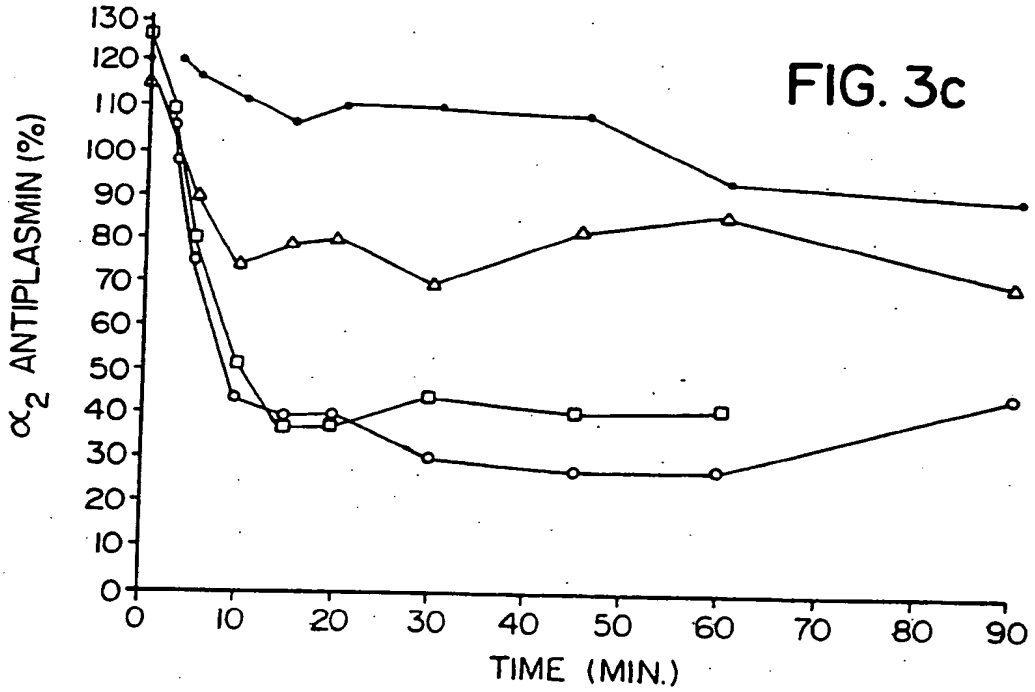



FIG. 3c

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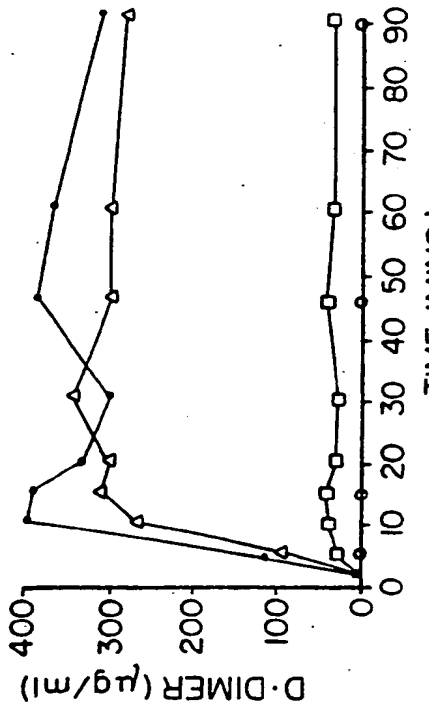


FIG. 4c

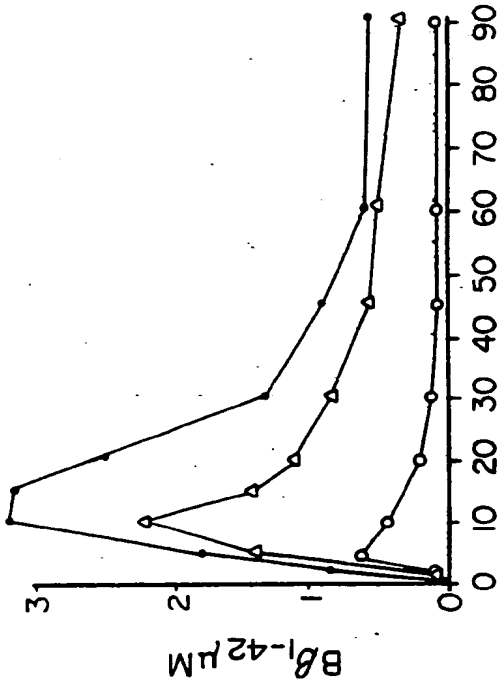


FIG. 4d

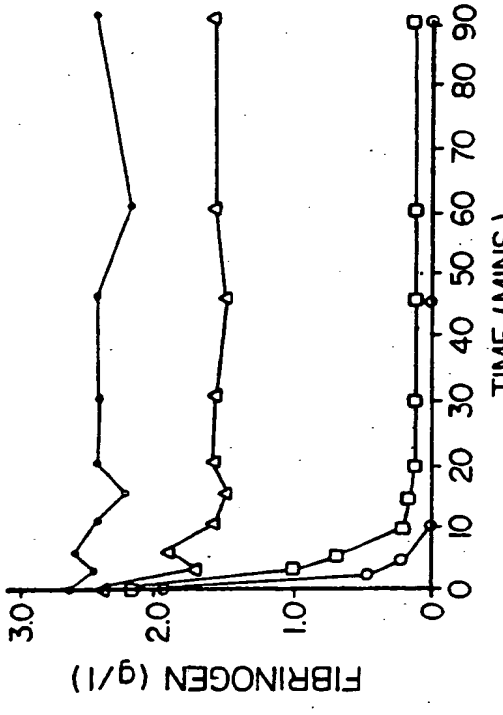


FIG. 4a

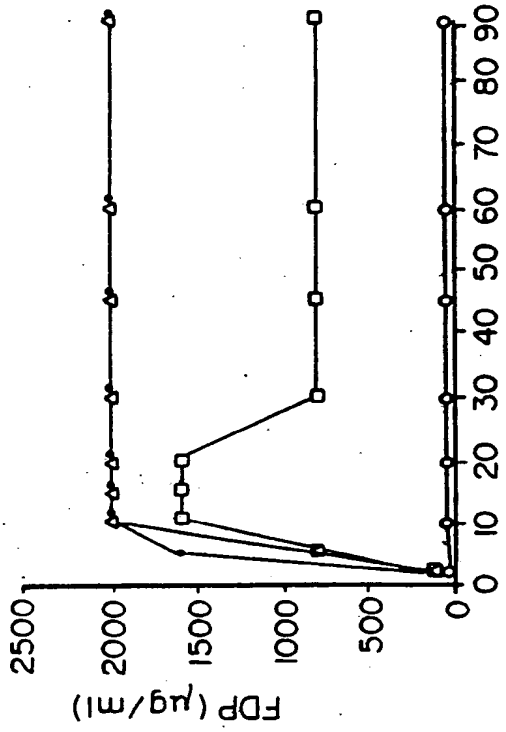


FIG. 4b

SUBSTITUTE SHEET

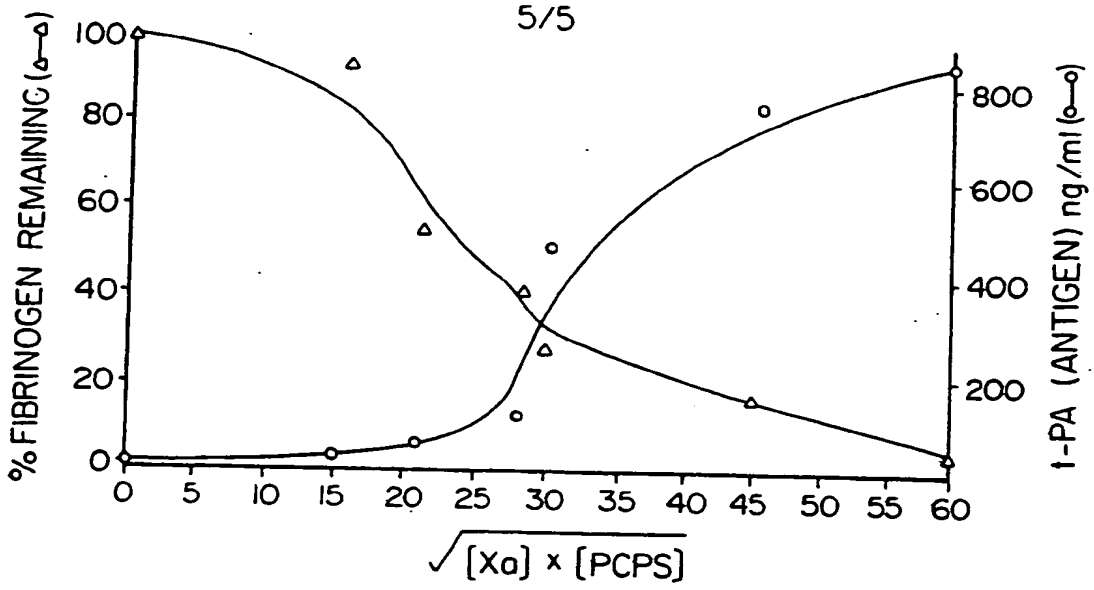


FIG. 5

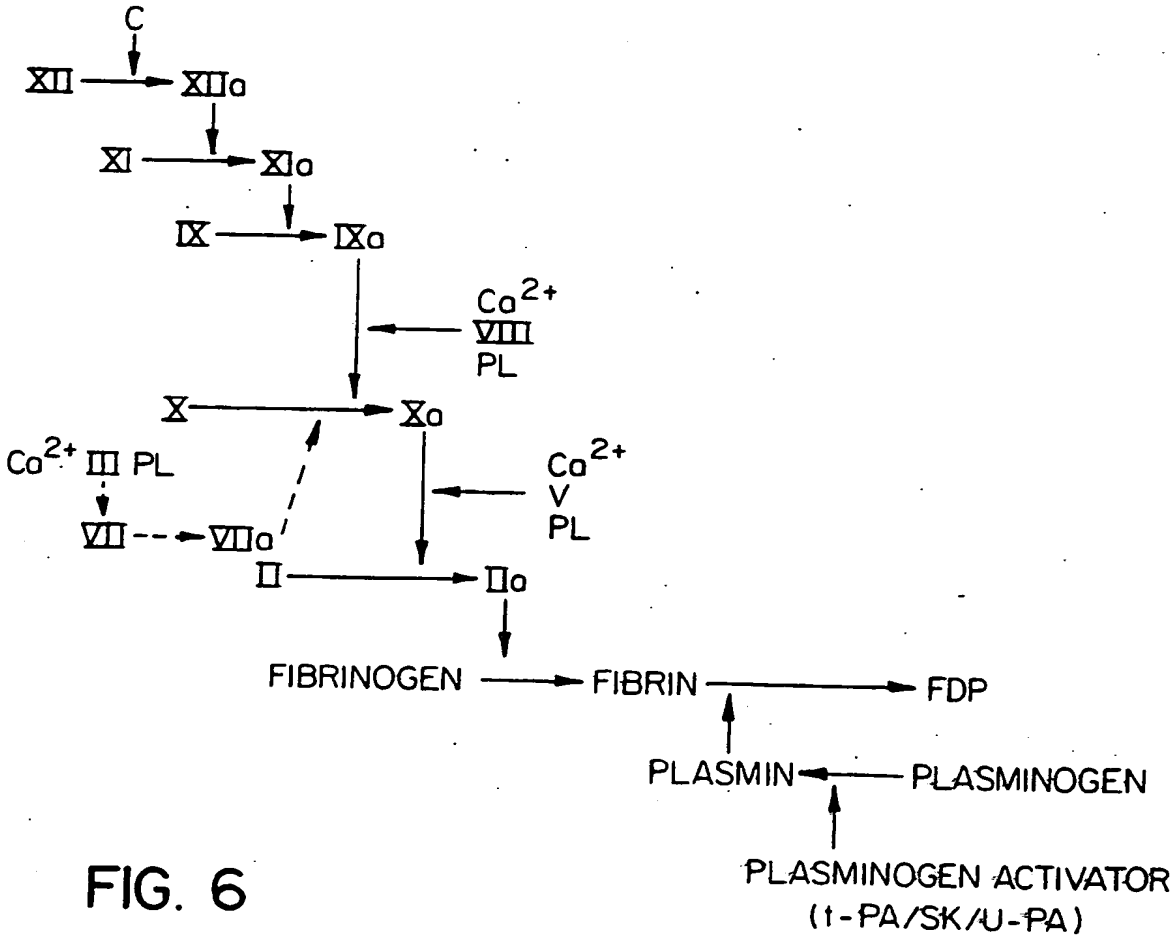


FIG. 6

# PATENT COOPERATION TREATY

## DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

issued pursuant to PCT Article 17(2)(a)(1)

| IDENTIFICATION OF THE INTERNATIONAL APPLICATION | APPLICANT's OR AGENT's FILE REFERENCE |
|---|---|
| | Q1311 |
| International Application No. PCT/CA 90/00234 | International Filing Date 25th July 1990 |
| Receiving Office RO/CA | Priority Date Claimed 14th August 1989 |

Applicant (Name)

QUEEN'S UNIVERSITY AT KINGSTON

### DECLARATION

This International Searching Authority hereby declares that no international search report will be established on the above-identified international application for the reasons indicated below. (1)

1. The subject matter of the international application relates to: (2)

a. [ ] scientific theories.

b. [ ] mathematical theories.

c. [ ] plant varieties.

d. [ ] animal varieties.

e. [ ] essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.

f. [ ] schemes, rules or methods of doing business.

g. [ ] schemes, rules or methods of performing purely mental acts.

h. [ ] schemes, rules or methods of playing games.

i. [X] methods for treatment of the human body by surgery or therapy.

j. [ ] methods for treatment of the animal body by surgery or therapy.

k. [X] diagnostic methods.

l. [ ] mere presentations of information.

m. [ ] computer programs for which this International Searching Authority is not equipped to search prior art.

2. The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out: (3)

a. [ ] the description.

b. [X] the claims.

c. [ ] the drawings.

comment:

Classification:- A 61 K 35/16
A 61 K 31/66

### CERTIFICATION

| International Searching Authority | Date of Mailing | Authorized Officer |
|---|---|---|
| ISA / EP | 17 OCT 1990 | MISS D.T. [illegible] |

Form PCT/ISA/210 (January 1985)